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Acute Phase Responses in Veal Calves Monitored by Sequential Determination of Serum Amyloid A, Haptoglobin and Mx Protein

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1. Summary

Production of sound animal-derived food with healthy animals is a major task of veterinary public health (VPH). However, in animal husbandry infectious diseases remain the leading cause of health impairment and economic losses. The interaction of invading pathogens with the host provokes inflammatory conditions. These are initiated by the hosts' acute phase response (APR). Based on this it was attempted to use acute phase proteins (APPs) as a tool for animal health monitoring. In this context elevated levels of haptoglobin (Hp) and serum amyloid A (SAA) reflect inflammatory conditions and elevated levels of Mx protein are indicative of an activated IFN-α/β system. The focus of the present study was a cohort of 3 age-matched groups of calves that were used to sequentially monitor serum levels of Hp and SAA as well as the cell-associated Mx protein. The cohort was managed to keep together at any time a younger and an older group of animals. Following arrival in the cohort the young calves invariably suffered from acute respiratory and/or gastrointestinal distress. This prompted repeated therapeutic interventions. Episodes of clinical disease were reflected by a distinct temporal rise of Hp and SAA as well as a prolonged expression of Mx protein in white blood cells. Virological and serological data additionally collected during the 6 months study disclosed the manifestation of several viruses, namely bovine rotavirus (BRV), bovine coronavirus (BCV), parainfluenza-3 virus (PIV3), bovine respiratory syncytial virus (BRSV), and norovirus (formerly Norwalk-like virus, NLV). BRV and BCV were prevalent throughout most of the observation period whereas BRSV was associated with a single outbreak. Faecal virus shedding and antiviral seroconversions continued beyond the post-arrival period and short lasting episodes of elevated levels of Hp and/or SAA but not Mx were recorded in a few individuals. With respect to Mx protein this indicated a state of hyporesponsiveness of the IFN-α/β system. In conclusion, this field study showed that assessment of APPs was exquisitely suited to highlight inflammatory conditions and an activated IFN-α/β system. By circumventing the necessity to trace specific pathogens, this tool has a clear prospect for VPH, enabling the surveillance of animal health.

2. Introduction

Infectious diseases of the digestive and respiratory tract remain the most important causes of morbidity and mortality in calves from birth up to several months of age (Radostitis et al., 1994). Acute diarrhea is a major concern with calves under one month of age and respiratory tract disease tends to affect animals at the age of 1 to 6 months. These conditions are most regularly provoked by multifactorial causes, namely prevalent pathogens, age and actual lactogenic immunity towards invading pathogens at time of marketing, transportation, changing social environment and animal housing as well as nutrition. The most important pathogens associated with diarrhea in calves are enterotoxigenic Escherichia coli, bovine rotaviruses (BRV), bovine coronaviruses (BCV), bovine viral diarrhoea virus (BVDV), Cryptosporidium sp., Salmonella spp., and coccidia (Eimeria spp.). Enzootic pneumonia, referred to as shipping fever, is also a multifactorial health problem. This condition is most regularly initiated by viruses, such as bovine respiratory syncytial virus (BRSV), parainfluenza-3 virus (PIV3), bovine herpesvirus 1 (BHV1), adenovirus (AdV), and reovirus (ReoV). The primary viral infections are usually aggravated at later times by secondary invading bacterial pathogens, mostly Mannheimia (Pasteurella) spp. (Radostitis et al., 1994). Collecting statistical data on calf morbidity is not as reliable as that on mortality because it depends on the producer's or veterinarian's clinical diagnosis, whether the animal will be treated for the illness. There is also a tendency of producers not to record every illness event. In this context several studies have indicated that the best available data on morbidity are based on treatment rates (Radostitis et al., 1994). The focus of another approach to monitor calf morbidity would be the acute phase response (APR) which is reflected by changing levels of acute phase proteins (APPs) in various body fluids, especially the serum (Baumann and Gauldie, 1994; Gruys et al., 1994). APPs are effector molecules of the innate immune system. Originating primarily from the liver, these molecules are up-regulated by the systemic action of proinflammatory cytokines, such as IL-1β, IL-6 and TNF-α, which, through action in the central nervous system, are additionally responsible for the animals' performance and productivity (Johnson and Langhans, 1997; Langhans, 2000) as well as fever and the general sickness behaviour (Johnson, 2002).

It has been reported that monitoring the plasma concentration of APPs, such as serum amyloid A (SAA) and haptoglobin (Hp), provides a valuable diagnostic information in cattle conditions that involve inflammation, infection, and trauma (Eckersall, 2000; Gruys et al., 1994). This tool has been adopted to discriminate between cattle with acute and sub-clinical or chronic inflammation (Alsemgeest et al., 1994; Eckersall et al., 2001; Horadagoda et al.,

1999; Karreman et al., 2000) and to detect cattle with clinical mastitis (Eckersall et al., 2001; Hirvonen et al., 1999). In an experimental model of bovine respiratory disease (BRD), induced by sequential infection of calves with BHV1 and P. haemolytica, (Godson et al., 1996) observed a temporal relationship between the onset of bacterial infection and increasing Hp serum levels. That study additionally disclosed that the concentration of Hp was a prognostic parameter. As outlined by (Heegaard et al., 2000) there is a general view that viral infections provoke little APRs if any, a fact that has been exploited in human clinical medicine to discriminate between viral and bacterial infections (Chieux et al., 1999, 1998; Forster et al., 1996; Halminen et al., 1997; Sasaki et al., 2002). Nevertheless, previous studies clearly showed rising levels of APPs in the serum of cattle during acute infections with BRSV, and BVDV (Ganheim et al., 2003; Heegaard et al., 2000; Muller-Doblies et al., 2002; 2004).

Virally infected stromal cells (Sen, 2001; Taniguchi and Takaoka, 2002) and activated dendritic cells, following binding of specific ligands to their Toll-like receptors (Aderem and Ulevitch, 2000; Akira, 2003; Underhill and Ozinsky, 2002) release a number of cytokines, notably IFN-α/β. In exposed cells IFN-α/β trigger antiviral defense mechanisms through the up-regulation and activation of several effector proteins, namely dsRNA-dependent protein kinase R (PKR), 2'-5'-oligoadenylate synthetase (OAS), and Mx proteins (Flohr et al., 1999; Kochs et al., 2002; Lee and Vidal, 2002; Staeheli, 1990). Both, virally activated PKR, provoking a general inhibition of cellular transcription, and OAS in conjunction with RNase L may contribute to apoptosis of virally infected cells. The Mx proteins are known to disable specific viral compounds so as to inhibit viral replication. IFN-α/β is also recognized as an important factor linking the innate and adaptive immune responses (Le Bon and Tough, 2002; Levy et al., 2003).

The goal of the present study was to monitor a cohort of milk-fed veal calves with respect to morbidity, the number of therapeutic interventions, and changing levels of the acute phase serum reactants SAA and Hp, as well as the WBC associated Mx proteins. Special emphasis was given to the Mx protein as a possible marker for ongoing viral infections. A survey of the manifestation of selected viruses was additionally conducted in an attempt to correlate any changing levels of acute phase reactants with such manifestations. The results obtained during the prospective six months study show that the parameters recorded were of value to mirror ongoing disease.

3. Materials and Methods

3.1. Cohort composition, animal management and study design

The study focussed on a cohort of veal calves held on a faculty owned teaching unit of milking cows. Based on existing regulations the cows milk cannot be marketed. For this reason it is necessary to permanently keep some 15 to 20 milk-fed veal calves of varying age to ascertain proper use of the milk. The actual situation necessitates the regular purchase of approximately 4 weeks old calves of differing origin and precludes the possibility to effectuate an all-in and all-out management system. The animals are held on deep straw in a well ventilated housing and offered a milk-based diet with free access to tap water. Calves are generally marketed for slaughter at the age of 20 to 25 weeks. Based on the sequential entry into the cohort the 33 animals included in the study were assigned to one of three groups (G1, G2, G3). The entire observation period encompassed a period of 26 weeks, starting in December 2001 and ending in May 2002. Samples for laboratory examinations were collected once a week from every calf present at a given time.

3.2. Clinical examinations

The animals were checked regularly by veterinary practitioners with respect to general health and skin condition, respiratory and abdominal status, nasal discharge, coughing, and diarrhea. The animals were medically treated according to actual requirements. During inspection for clinical signs rectal temperature was weekly recorded. Therapeutic interventions, routinely necessary during the post-arrival period, encompassed antibiotics and/or antiphlogistics. During week 9 (W9) the animals of G2 were treated with an ivermectine (Doramectin®) due to pediculosis. From W6 to W23 the body weight of the calves from G2 and G3 was additionally recorded.

3.3. Collection and processing of clinical samples for laboratory examination

Jugular blood samples were taken on a weekly basis. Sera recovered from coagulated samples were stored at –20°C until used for the determination of SAA, Hp, and antiviral antibodies. The number of leukocytes in anticoagulated (EDTA) blood samples was determined using an automated procedure (CELL-DYN®3500, Abbott Laboratories). White blood cells (WBC), isolated from anticoagulated blood samples, were used for determination of WBC-associated Mx proteins (Muller-Doblies et al., 2002, 2004). Briefly, following lysis of red blood cells with buffered ammonium-chloride the resulting WBC were washed once with PBS,

suspended in SDS-PAGE sample buffer, solubilized by heating for 3 min at 95°C, and kept for short-term storage at –20°C.

Faecal samples were collected from the rectum and equal aliquots from 4 to 6 animals were pooled and processed the same day for virus assay by RT-PCR. Pooled faecal samples were diluted (1:4) with PBS containing 0.04% of sodium azide for long-term storage at 4°C, centrifuged, and 140 µl aliquots of the supernatant used for viral RNA isolation.

3.4. Determination serum levels of SAA and Hp

Serum samples were evaluated for Hp and SAA with commercially available test kits (PHASE™ Hp and PHASE™ SAA, Tridelta Development Ltd., Greystones, Ireland), using the supplied calibrators and following the instructions of the manufacturer as described previously (Muller-Doblies et al., 2004). PHASE™ SAA is a monoclonal antibody-based capture ELISA, whereas PHASE™ Hp is based on the peroxidase activity of haemoglobin-haptoglobin complexes at low pH-values. Results, recorded with a photometer (450 and 630 nm, respectively), are expressed in µg/ml of serum. The tests cut-off value were determined to be 26 µg/ml and 6 µg/ml for Hp and SAA, respectively.

3.5. Determination of Mx protein levels in WBC

Immunoblotting for Mx proteins was effectuated following discontinuous SDS-PAGE of the proteins from 50'000 to 100'000 WBC equivalents and using the anti-Mx monoclonal antibody M143 (Flohr et al., 1999) in conjunction with chemiluminescence (Muller-Doblies et al., 2002, 2004). Quantification of Mx signals was achieved by densitometry using a Chemilmager™ 5000 (Alpha Innotech Corp., San Leandro, CA) and setting the positive control as a relative 100% value. The respective positive and negative controls were obtained by culturing MDBK cells over night in the presence or absence of 1000 IU/ml of rIFN-αB/D and analysing 50'000 cell equivalents by immunoblotting (Muller-Doblies et al., 2002, 2004).

3.6. Determination of antiviral serum antibodies

Serum antibody titers to BRSV, BCV, and PIV-3 were determined with commercially available ELISA kits (Svanovir, Svanova, Uppsala, Sweden) by using the kit-contained calibrators and following the instructions of the manufacturer. The optical density (OD) obtained in the presence of the control antigen were deduced from the OD obtained in the presence of the viral antigen to obtain ΔOD. A seroconversion was based on the calculation [(ΔOD week$_x$ / ΔOD week$_{x-2}$) x 100%] and considered as such for values of ≥ 130%. This

approach was based on reports showing that seroconversions to various viruses can be reliably determined with acute and reconvalescent sera in ELISA tests by using single serum dilutions (Collins et al., 1985; Graham et al., 1997, 1998). To scope with some borderline cases a definite seroconversion was attested only if antibody levels continued to increase for at least two consecutive two-weeks periods.

3.7. Molecular detection of faecally shed viral pathogens

RNA was extracted from 140 µl aliquots of the pooled and pre-diluted faecal samples by using the QIAamp® Viral RNA Mini Kit (Qiagen, Basel, Switzerland). Purified RNA was collected in 50 µl water and used for virus-specific RT-PCR. The primers used targeted BRV, BCV, and NLV. Primers were taken from published data. BRV: antisense 5'-GGTCACATCATACAATTCTAATCTTAAG-3', sense 5'-GATATAACAGCTGATCCAACAAC-3', product length 208 bp (Leisinger and Metzler, 1997); BCV: antisense 5'-GGCCTAACATACATCCTTCC-3', sense 5'-ACCACCAGTTCTTGATGTGG-3', product length 455 bp (Fukutomi et al., 1999); NLV: antisense 5'-TCATCATCACCATAGAAAGAG-3', sense 5'-ATACCACTATGATGCAGATTA-3', product length 326 bp (van Der Poel et al., 2000). Reference virus strains, used for control purposes, were from the institutes repository (A. Metzler, unpublished). For RT of specified viral targets the RNA templates (10 µl) were prepared by heating for 10 min at 95°C and subsequent refrigeration at 4°C. This was followed by adding 10 µl RT-mix (Cat. no. A3500, Catalys AG, Wallisellen, Switzerland) to give final concentrations of 1 x RT buffer, 2.5 mM $MgCl_2$, 1 mM (each) dNTPs, 20 U RNasin, 14 U AMV-RT, and 1.2 µM antisense primer (Microsynth, Balgach, Switzerland). RT was effectuated in a thermocycler (Gen Amp 2400 PCR System, Perkin Elmer Corp.) at 42°C for 60 min and terminated by a heating step (94°C for 5 min). RT was followed by adding 30 µl of a PCR-mix (Cat. no. M7660, Catalys AG) to give final concentrations of 1 x PCR buffer, 2.5 mM $MgCl_2$, 1.25 U Taq polymerase, and 0.8 µM sense primer. This mixture was initially heated to 94°C for 2 min, followed by 35 rounds of thermocycling (30 sec at 94ºC, 60 sec at 49°C, 60 sec at 72°C) and finalizing by a 10 min elongation step (72°C) before cooling to 4°C. Amplified PCR products (12 µl) were electrophoresed in 1.5% agarose gels (with added 0.5 µg/ml ethidium bromide) and photographed under UV-illumination.

4. Results

4.1. Group Dynamics and Health Status of the Calves

This prospective study aimed to monitor morbidity in a cohort of milk-fed veal calves as reflected by the need for therapeutic interventions and changing levels of APPs, namely SAA, Hp and Mx. The observation period started in December 2001, lasted 26 weeks and involved a total of 33 calves. Based on the sequential entry into the cohort the animals were assigned to one of three groups, G1, G2 and G3 (table 1). G1 consisted of 10 calves (C1 to C10), initially aged from 15 to 20 weeks. It was noted that these near to marketing animals did not necessitate any therapeutic intervention. The animals were sequentially sold for slaughter (sfs) as indicated. The latest marketing was effectuated during week 10 (W10). An additional animal (C11) was kept in a separate pen in the animal housing for a limited time period (W6 to W9). This animal was assigned to G1 for completeness of the records. At the beginning of the study (W1) 10 newly purchased, 3 to 4 weeks old calves (C12 to C21) were introduced into the cohort. These animals, assigned to G2, suffered from respiratory and gastrointestinal distress during the first two weeks post-arrival, necessitating repeated therapeutic interventions (T). One calf (C16) died during W1 due to a peracute lethality of undetermined etiology. This animal was replaced during the following week by C22. Since the animals were provided with antibiotics and anti-inflammatory therapeutics, long lasting high fever (up to 41°C) was seen in a few cases only. Some animals, namely C17, C20 and C21, displayed more than one clinical episode, necessitating additional therapeutic interventions. During W9 all animals of G2 had to be treated with an avermectine (t) due to pediculosis. C21 was removed from the group (R) during W11 due to a ruminal drinking condition. An additional animal (C18) died during W14 due to an incurably progressive disease. Post-mortem necropsy revealed a ruptured ulcer of the abomasum and a chronic bronchopneumonia. Four of the G2 calves were ready for marketing in W20. The other animals of this group remained in the cohort up to the end of the study.

With completion of marketing the animals from G1 (W10) the cohort was replenished during W11 with 7 newly purchased replacer calves. These animals were assigned to G3. Calf C29 broke a leg and was removed from the cohort during the first week post-arrival. G3 was completed during W12 with 4 additional calves. With one exception (C33) all the individuals in G3 necessitated partly multiple therapeutic interventions. Compared to G2 the manifestation of clinical distress in G3 was somewhat delayed and the severity of the disease appeared less important (data not shown).

The mean daily weight gain observed with G2 and G3 calves was 0.86 kg and 0.82 kg, respectively. In summary, of the 33 calves that constituted the cohort (table 1), there were 2 lethalities (†), 2 animals had to be removed from the cohort for medical reasons (R), and 20 animals had to be medically treated with antibiotics and/or anti-inflammatory drugs (T) for partly extended periods.

4.2. Manifestation of viral pathogens

This part of the study aimed to possibly find a correlation between apparent morbidity, therapeutic interventions or altered expression of APPs and the manifestation of selected viral pathogens. Being aware of a great diversity of likely prevalent viral and non-viral pathogens, the respective experiments focussed exclusively on BRV, BCV and NLV as enteropathogens, and BCV, BRSV and PIV-3 as pathogens of the respiratory tract. BRV was shed in the cohort throughout most of the study period (table 2). Shedding of this virus tended to be more consistent in younger animals than in older ones. Sporadic shedding of NLV was observed with G1 and G3 animals. Of special interest was the observation that BCV was exclusively and most consistently shed in the faeces of G3 animals. To conclude, shedding of BRV and BCV was virtually manifest within the cohort throughout the entire study period. This means that the cohort was permanently exposed to these viral pathogens.

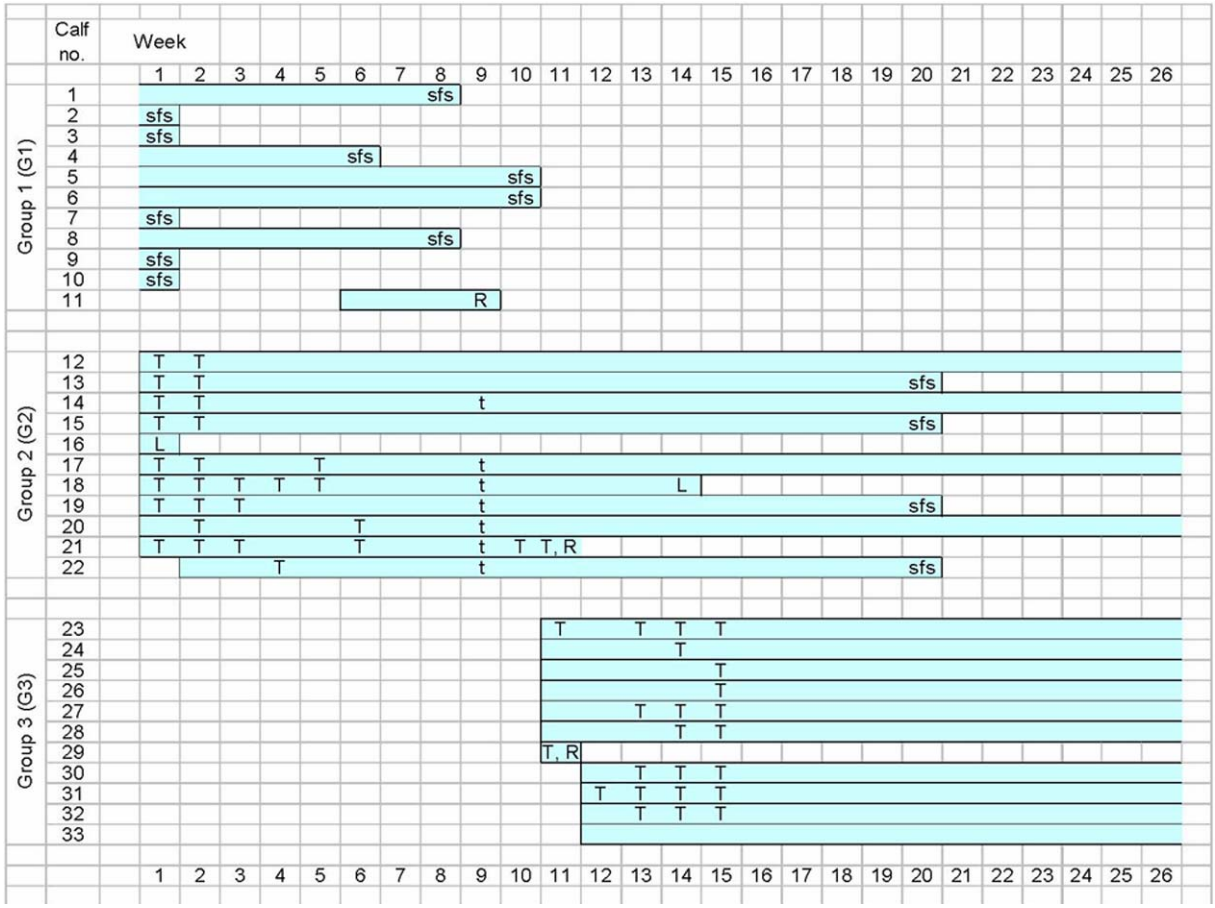

table 1: A cohort of three age-matched groups of calves (G1, G2, and G3) was used to prospectively record individual clinical distress, therapeutic interventions and the time-point and reason for removal from the cohort. The calves were purchased at the age of 3 to 4 weeks and stayed in the cohort for up to 26 weeks. Note: boxes closed to the left, newly introduced into the cohort; boxes closed to the right, removed from the cohort.
Abbreviations: sfs, sold for slaughter; T, therapeutic intervention; t, treatment with Doramectin®; R, removed from the cohort for medical reasons; †, lethality.

In the context of the respiratory pathogens BCV, PIV-3 and BRSV it was noted that none of the animals from G1 exhibited a seroconversion. Seroconversions towards BCV became evident in G2 in 5 of 10 animals during W3. Rising antibody titers to this virus were partly prolonged and, of considerable interest, repeatedly apparent in some of the animals. This is exemplified by C15 which showed a first seroconversion from W3 to W12 and a second one from W16 to W19. Of interest is the notion that all calves from G2 mounted a seroconversion towards BCV within 10 weeks post-arrival without concomitant faecal shedding of the respective virus. Considering that it takes from 2 to 3 weeks from primary infection to a measurable seroconversion it seems likely that most animals from G2 were acutely infected with BCV during the first week post-arrival. This virus then persisted in the cohort until the end of the observation period (see G3 animals). A single calf (C21) seroconverted with respect to PIV-3 (W5 to W8). Of the 8 animals from G2 that remained beyond W14 in the

cohort five were observed to seroconvert towards BRSV.

Calves from G3 were observed to faecally shed BCV during W1 post-arrival. This indicated an acute infection with this virus that was either entering the cohort with one of the newly purchased animals (possibly C27) or acquired from virus shedding G2 animals. Ultimately 8 of the 10 animals from G3 exhibited a BCV-directed seroconversion. The notion of 2 non-seroconverters (C25 and C26) indicated that individual animals may resist infection with BCV for undetermined reasons. A single calf of G3 (C30) seroconverted towards PIV-3 (W18 to W23). This same animal was the first one of the cohort to seroconvert towards BRSV (W14). It seems likely that C30, entering the cohort during W12 in a state of acute infection with BRSV, would be expected to show a seroconversion within 2 weeks, i.e. by W14. It is anticipated that the BRSV outbreak originated from this animal. The long lasting seroconversion rates towards BRSV observed in this and some other calves indicated the hosts' long lasting immunological interaction with this virus, i.e. a state of viral persistence.

Eight animals from G2 and G3 did not seroconvert towards BRSV. It was retrospectively noted that these animals upon arrival invariably displayed elevated (cholostral) antibody titers towards BRSV (ΔOD >1.2). This contrasted with the respective findings of the seroresponders (ΔOD <1.2). With respect to PIV-3 the calves generally displayed elevated antibody levels upon arrival that tended to fade off during the weeks to follow. A similar picture was not apparent with respect to BCV. This would be in agreement with the long lasting manifestation of this virus in the cohort.

In conclusion, during the study the cohort was exposed to several viruses, namely BRV, NLV, BCV, PIV-3 and BRSV. BRV and BCV were shed within the cohort during most of the study period, whereas BRSV was found to be manifest during a single outbreak. In the context of BCV the obvious discrepancy observed with respect to the seroconversions in G2 and G3 and the exclusive faecal shedding of BCV by G3 animals could possibly be explained by the manifestation of a BCV biotype with differing organ tropism.

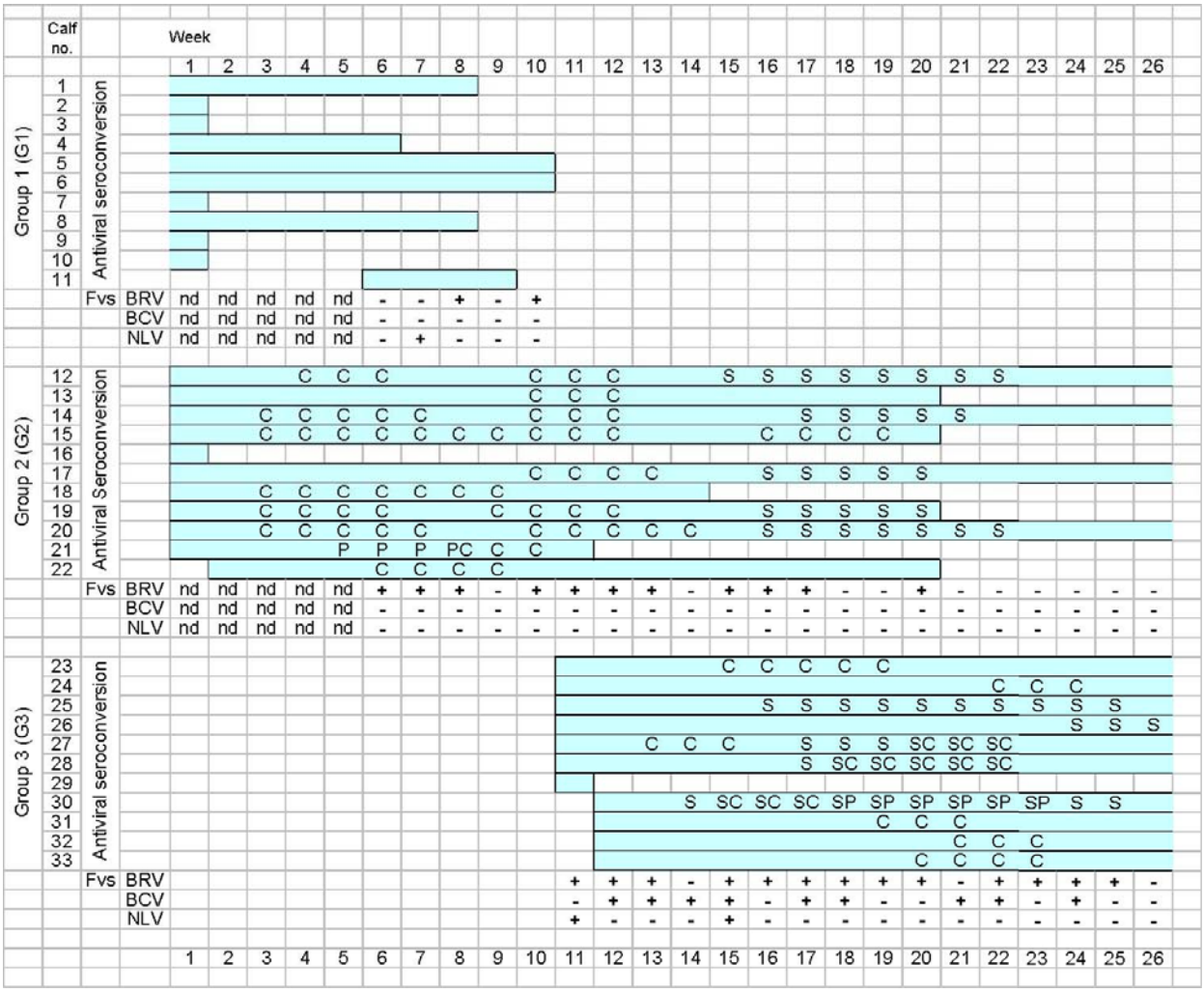

table 2: Individual antiviral seroconversions recorded by indirect ELISA and fecal virus shedding (Fvs) within groups of animals as revealed by RT-PCR. A seroconversion was defined as an elevation of the specific reactivity during any two weeks interval in excess of 30% as follows: [ΔOD week x / ΔOD week (x-2)] x 100%. Seroconversions towards bovine coronavirus (C), parainfluenza-3 virus (P), and bovine respiratory syncytial virus (S) are indicated. Viral shedding focussed on group A bovine rotaviruses (BRV), bovine coronavirus (BCV), and norovirus, previously known as Norwalk-like virus (NLV).
Abbreviations: nd, not done; +, positive finding; -, negative finding.

4.3. Changing serum levels of SAA and Hp mirror ongoing inflammatory disease

Hp and SAA are known as major bovine APPs. It was therefore of interest to focus on these proteins as a tool to disclose the manifestation of inflammatory conditions. Fig. 1A and 1B depict the individual serum levels of Hp and SAA observed with G1, G2 and G3 animals. There is evidence of a great individual variability of the recordings, indicating the manifestation of inflammatory states of varying severity. Mean individual levels of Hp and SAA recorded never exceeded 300 µg/ml and 60 µg/ml, respectively. C21, which had to be removed from the cohort during W11 due to a ruminal drinking condition, terminally displayed the highest Hp level observed during the entire study (1500 µg/ml). The calves

from the older age group G1 remained without clinical distress until sold for slaughtering. These animals were mostly observed to display near baseline serum levels of Hp and SAA (Fig. 1A, B). A single animal (C4) was noticed during W4 to display a short lasting APR episode. In G2 and G3, and with respect to Hp and SAA levels, there were 5 and 3 animals that were identified as low responders (C12, C24, C25, C26, and C33, respectively C12, C17, and C18). In these multiply tested animals Hp and SAA levels never exceeded 300 and 60 µg/ml, respectively.

During the post-arrival period most of the G2 animals displayed at times distinctly rising levels of Hp and SAA (Fig. 2A, B). This was obviously correlated with episodes of clinical disease necessitating therapeutic interventions. Individually this response was more or less pronounced, most likely depending on the single animals' pathological conditions. This was reflected by multiple peaks of the mean observed with both APPs. In any case, serum levels of Hp and SAA tended to vanish with time towards baseline values. This was paralleled by gradually improving health conditions of the calves. On occasion single animals displayed short periods of elevated serum levels of Hp and/or SAA, indicating the manifestation of clinically inapparent inflammatory episodes. In this respect it was of interest to note that individually elevated levels of Hp were not necessarily paralleled by elevated levels of SAA and vice versa. With respect to Hp and SAA the G3 animals displayed a similar reaction pattern as seen with G2 animals. One obvious difference was seen with SAA, which appeared elevated in G3 as early as during the first week (W11) post-arrival.

At this point it should be noticed that the therapeutic interventions with antibiotics and antiinflammatory compounds during the post-arrival period were likely to interfere with the individual APRs. These confounding interventions have to be considered when assessing APRs of both the individual animals as well as the groups.

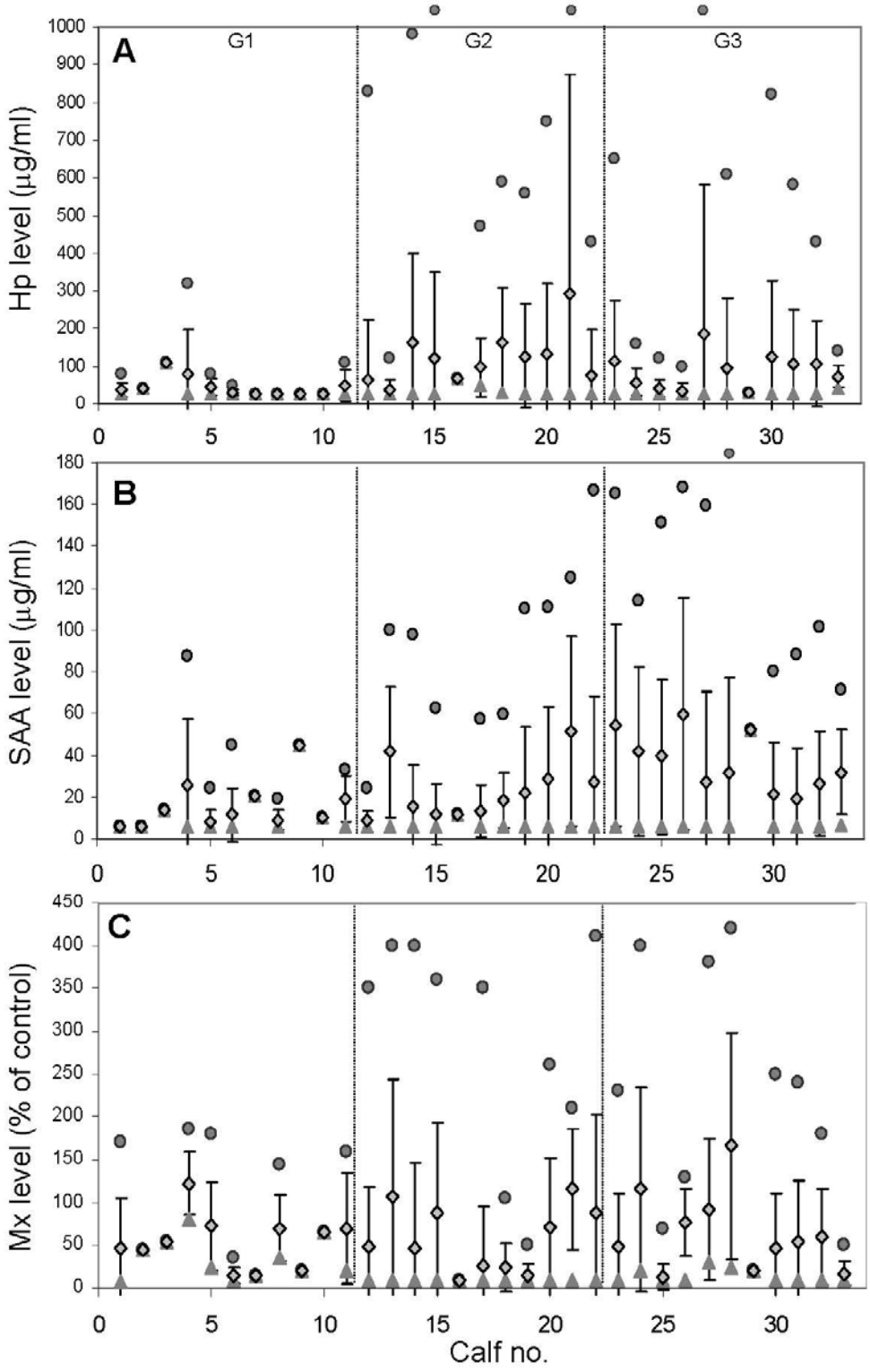

figure 1: Individual levels of APPs displayed in three age-matched calf groups, G1, G2 and G3, weekly recorded during the animals presence in the cohort. Shown are the individual means (diamonds) ± se, including maximum (filled circles) and minimum (filled triangles) recordings. A, levels of haptoglobin (Hp); B, levels of serum amyloid A (SAA); C, levels of white blood cell-associated Mx protein (Mx). Note the different scale of the ordinates.

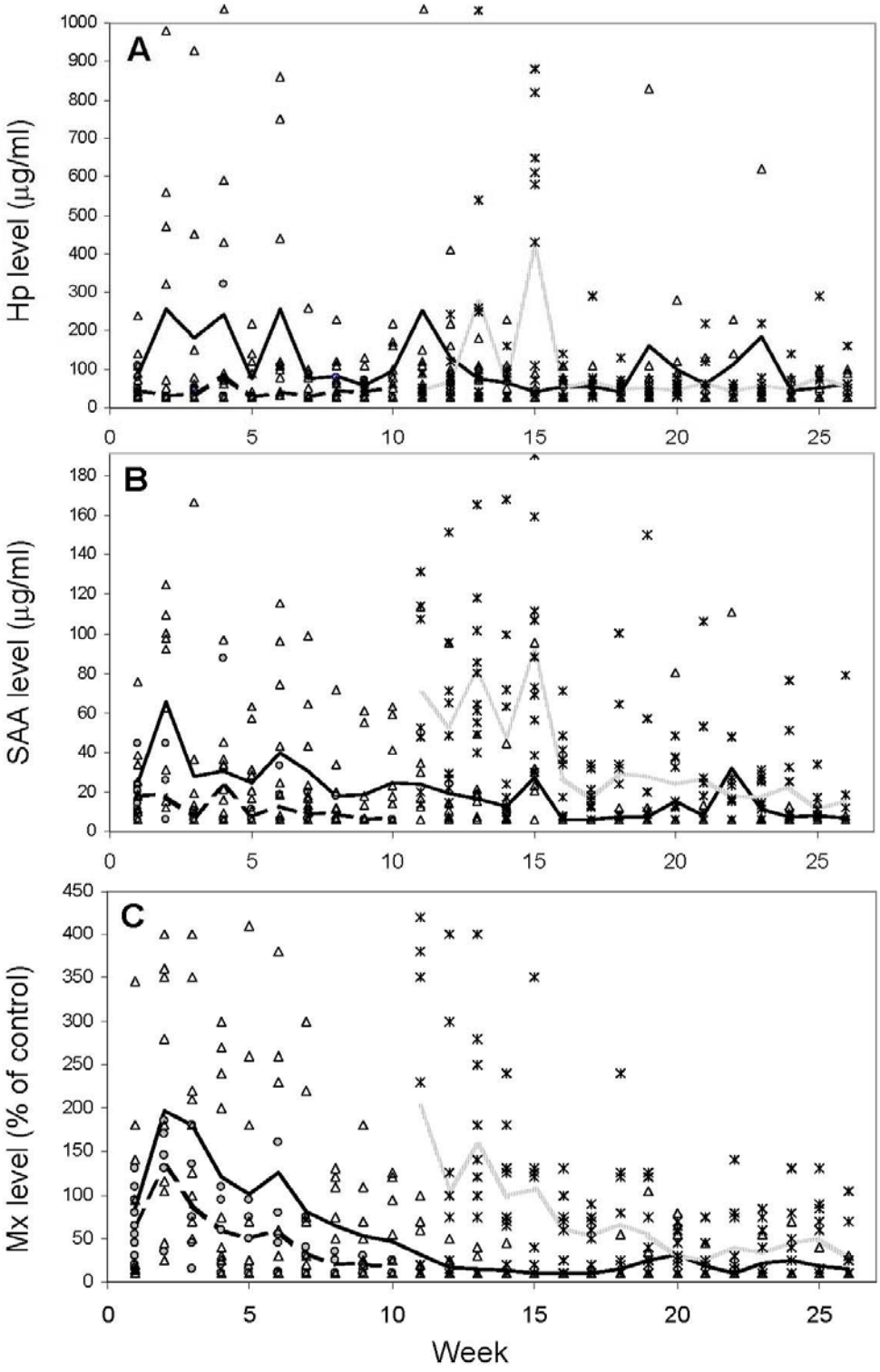

figure 2: Acute phase protein profiles of three age-matched calf groups, G1 (broken line), G2 (black line) and G3 (grey line), recorded during the groups presence in the cohort. Shown are the mean of the groups and individual reactivities over time. Circle, G1 animal; triangle, G2 animal; asterisk, G3 animal. A, levels of haptoglobin (Hp); B, levels of serum amyloid A (SAA); C, levels of white blood cell-associated Mx protein (Mx). Note the animal groups presence in the cohort: G1 from week 1 (W1) to W10, G2 from W1 to W26, and G3 from W11 to W26.

4.4. Mx protein levels in WBC mirror the activation of the IFN-α/β system

Besides the genes of PKR and OAS, the Mx gene is one of the well established targets that is up-regulated in cells following exposure to and down-stream signalling of IFN-α/β. It was therefore of interest to determine as to whether the Mx protein could be used as a novel tool for animal health monitoring. A semi-quantitative determination of Mx proteins in WBC was achieved by immunoblotting with a monoclonal antibody (M143) and densitometric scanning of the chemiluminescence signals (Fig. 3). This method revealed at times the manifestation of two closely migrating immuno-reactive Mx proteins both with an apparent molecular weight of approximately 75 kDa. This corresponds well with protein data on bovine Mx1 and bovine Mx1-a reported by others (Ellinwood et al., 1998). Fig. 1C depicts the individual Mx protein recordings of the cohort. There was a significant variability of the individual reactivities, similarly seen with Hp and SAA. Mean individual levels of Mx protein remained invariably below the value of 175% when compared with the 100% positive control (Fig. 3). With G1 animals maximal levels of Mx protein never exceeded the value of 200%. This contrasted with the findings of some G2 and G3 animals, showing at times Mx protein levels in excess of 400% (Fig. 1C). Within the cohort there were 4 multiply examined animals (C6, C19, C25, and C33) that were assigned the status of "low responders".

Several animals from G1 displayed short lasting elevated levels of Mx protein during the post-arrival period of the G2 calves (Fig. 2C). This could possibly reflect the subclinical manifestation of a horizontally transmitted, facultative pathogen. In the absence of overt disease this could be explained by the activation of the IFN-α/β system by a non-inflammatory condition, such as a subclinical virus infection. During the post-arrival period the G2 animals showed a rising mean level of Mx protein. After reaching a plateau during W2 the reactivity appeared elevated until W10. After this time point the Mx protein recordings remained close to baseline levels. The Mx protein recordings observed with G3 animals indicated again that most of these animals were entering the cohort in a state of acute infection, i.e. an activated IFN-α/β system. With this animal group the Mx protein levels recorded also remained elevated for 10 weeks.

In summary, the results of this study enable a better understanding of morbidity in conventionally reared veal calves. Bringing newly purchased young calves into a cohort of older animals was invariably followed by the manifestation of respiratory and/or gastrointestinal distress. This was especially pronounced in the young and necessitated partly prolonged therapeutic interventions. In the newly arriving animals the post-arrival period was characterized by temporary rising levels of APPs, including Hp, SAA and Mx protein. This

allowed to depict the manifestation of inflammatory conditions and the activated IFN-α/β system and was therefore of value to monitor morbidity. Elevated levels of Hp and SAA could be followed for up to 6 weeks post-arrival. This contrasted with the Mx protein which displayed a diagnostic window up to 10 weeks post-arrival. Virological and serological data additionally collected during the study confirmed that the cohort was exposed to multiple viral pathogens, namely enteropathogenic BRV, BCV, and NLV as well as the respiropathogenic BRSV, PIV3 and possibly a respiropathogenic variant of BCV, i.e. Bovine Respiratory Coronavirus (BRCV).

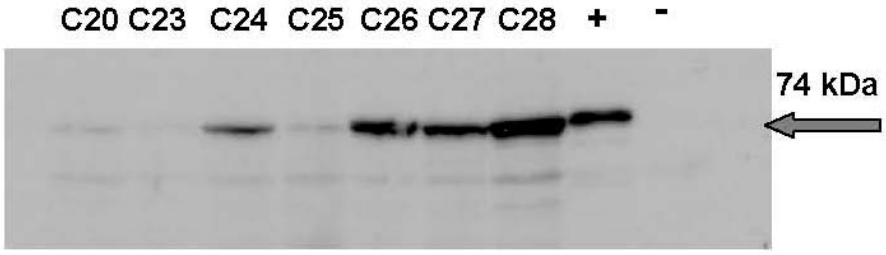

figure 3: Representative results of an immunoblot assay used to detect Mx protein in white blood cells (WBC) of selected calves as revealed with a monoclonal antibody. Data shown were obtained with WBC recovered during week 18. Abbreviations: C, calf number; +, positive control; -, negative control. Compared to the positive control (100%) the densitometric reactivities of the calves 20 and 23 to 28 were 10%, 10%, 80%, 10%, 125%, 120%, and 240%, respectively. Note the Mx reactivity of C28, showing two closely migrating protein bands. These virtually represent the allelic forms of bovine Mx protein as predicted from nucleotide sequence data (Ellinwood et al., 1998).

5. Discussion

The aim of the present study was to elucidate the feasibility to monitor morbidity in animal husbandry by simultaneous recording therapeutic interventions and changing levels of APPs, notably Hp, SAA and the cell-associated Mx protein. Special emphasis was given to the Mx protein as a possible specific marker for ongoing viral infections. The focus of the field study was a cohort of milk-fed veal calves consisting of differently aged animals that originated from different providers. Such an animal rearing system is known to be associated with a high risk of morbidity and mortality (Radostitis et al., 1994). Nevertheless, in Switzerland this kind of animal husbandry is very common. Specifically, on the teaching unit under study this type of animal keeping is dictated by the obligation to use the cows daily produced milk as a feed for veal calves. The cohort studied was followed for a period of 26 weeks (table 1). It consisted of 33 animals that were assigned to one of three basically age-matched groups (G1,

G2 and G3). These were managed to keep together at any time a younger and an older group, first G1 and G2, followed by G2 and G3. During the study there were 2 lethalities, 2 animals had to be removed from the cohort for medical reasons, and 20 animals had to be treated with antibiotics and/or anti-inflammatory compounds for partly extended periods. Morbidity was invariably prominent in the newly introduced young calves and not overt in the older animals. As a matter of fact, there was only one individual within the cohort (C33, see Fig. 1) that was not necessitating any therapeutic intervention during the early post-arrival period. Upon feeding calves with a milk-based diet one would expect a daily weight gain of 1.2 to 1.5 kg on the average (Kirchgessner, 1970). This goal was neither achieved with the animals from G2 (0.86 kg mean daily weight gain) nor with those from G3 (0.82 kg mean daily weight gain). To conclude, the cumulative economic losses within the cohort were substantial, involving not only mortalities, medical drugs and the impaired weight gain but also the hands-on of the caregiver and the veterinarian.

Young calves are at risk with a great number of infectious agents, including viruses and non-viral pathogens. This risk is likely influenced by multiple factors, such as timely ingestion of suitable colostrum, actual age, immunization status, animal housing (bedding, ventilation), exposure to prevalent infectious pathogens, management and food as well as transportation and social stress. Upon arrival in the cohort the young animals regularly suffered from morbidity. As could be expected with animals of differing origin and disposition, the observed morbidity and APRs run an individual course. Nevertheless, the post-arrival period was invariably paralleled by a distinct APR within the groups which, in the case of Hp and SAA, lasted up to 6 weeks. This indicated that the respective animals were suffering from inflammatory conditions for extended periods. With a view to the observed temporal rising levels of Hp and SAA it should be recalled that the adopted treatment regimens (antibiotics and anti-inflammatory drugs) were likely to have influenced the manifestation of these diagnostic and prognostic factors (Gruys et al., 1994; Hulten et al., 2003). Ultimately, the specific trigger(s) for the observed APR's could not be evidenced, a fact that has to consider the likely manifestation of a multitude of prevalent infectious agents. This and the observation that individually changing levels of Hp and SAA were not necessarily correlated to each other remain to be elucidated further. Together, these observations are likely to reflect the individually divergent fine-tuning of the APR.

Levels of Mx protein detected in WBC during the post-arrival period followed a similar course as seen with Hp and SAA. However, with the Mx protein there was notice of two peculiarities. It was first observed that elevated post-arrival levels of the Mx protein lasted up

to 10 weeks thus providing a comparatively broader diagnostic window. The second observation indicated that some animals from G1 were mounting an IFN-α/β response, as seen by rising Mx protein levels. This was an indication for the possible manifestation of a horizontally transmitted, subclinical infection of undetermined aetiology.

It is generally accepted that expression of the Mx proteins is under the strict control of IFN-α/β and it was anticipated until previously to be exclusively triggered by viruses or dsRNA (Sen, 2001; Staeheli, 1990; Taniguchi and Takaoka, 2002). However, there is growing evidence, that the IFN-α/β system is also activated in dendritic cells through interaction of their Toll-like receptors with bacterial LPS (Akira, 2003; Underhill and Ozinsky, 2002). Even by considering the likely existence of species-specific differences (Murtaugh et al., 1996) it must be concluded at the present, that activation of the IFN-α/β system is not restricted to viral infections (Hertzog et al., 2003). The present study disclosed a partly long-term manifestation of several viral pathogens, namely BRV, BCV and BRSV. The specific causes for the activation of the IFN-α/β system observed during the post-arrival periods remained undetermined for obvious reasons. This activation, as reflected by elevated levels of Mx protein, was apparent from the first week post-arrival and lasted up to 10 weeks. In view to this it was of interest, that the IFN-α/β system remained quiescent both during the outbreak of BRSV and the continued manifestation of BCV and BRV. This was especially evident by considering the corresponding lack of reactivity of the G2 animals (table 2). Such a behaviour is not surprising in the light of growing evidence showing that numerous viruses have evolved mechanisms to subvert the IFN-α/β system (Garcia-Sastre, 2002; Levy and Garcia-Sastre, 2001). In view of the anticipated high prevalence of a multitude of viral and non-viral pathogens during the study there remain unanswered questions relating to the long-term regulation of the innate immune system. Since the animals were successively running non-inflammatory conditions, the focus will likely be the IFN-α/β system. To subvert the IFN-α/β system the viruses have, through the action of some of their gene products, four possibilities. These are either inhibition of induction, signalling and signal-transduction, or interference with induced effector proteins. Recent evidence with BRSV shows in virally infected cells an inhibition of signal-transduction (Schlender et al., 2000). In the case of BRV and BCV the situation warrants to be further elucidated. To conclude, the observed lack of a systemic activation of the IFN-α/β system during the late post-arrival period, i.e. the lack of elevated levels of Mx protein in WBC, must be explained by an interference with IFN-α/β signalling. As an established enteropathogen BCV is assigned to a single viral species. Recent evidence shows that this virus is often involved in BRD (Storz et al., 2000a, 2000b) and simultaneously

shed in faeces and nasal secretions (Hasoksuz et al., 2002; Heckert et al., 1990; Niskanen et al., 2002). This is indicative of the possible existence of differing viral biotypes (Cho et al., 2001; Chouljenko et al., 2001; Fukutomi et al., 1999; Hasoksuz et al., 1999), an anticipation that needs further experimental exploitation. Seroconversions observed in G2 and G3 animals was not dependent on the calves age, yet faecal shedding was only observed with G3 animals. It could be anticipated that concurrent infections with additional pathogens in G2 animals was leading to a lowered faecal shedding of BCV that was below the detection limit of the RT-PCR assay. Such an argumentation is in line with observations of others (Niskanen et al., 2002) showing that concurrent infection with BVDV was a cause to drastically reduce the amount of fecally shed BCV. Another finding of relevance with BCV was the repeated seroconversion observed with some of the animals. This previously described phenomenon (Heckert et al., 1990) indicates that recovery from BCV infection is not followed by a long lasting immunity. Finally, the observation that high titered (passively acquired colostral) antibodies to BRSV reflected a state of protection against infection with this virus, may be of prognostic value.

To summarize, the early post-arrival manifestation of acute febrile disease among groups of age-matched veal calves was promptly paralleled by rising levels of APPs. In the case of Hp and SAA this response could be followed up to 6 weeks, i.e. up to the virtual disappearance of overt disease and irrespective of the long-term manifestation of infections with BRV, BCV and BRSV. Levels of expressed Mx protein, which is under the control of IFN-α/β, initially showed a similar kinetic as seen with Hp and SAA. However, elevated levels of this cell-associated protein could be demonstrated up to 10 weeks post arrival. This comparatively long lasting diagnostic window, also seen during the course of a transient infection with BVDV (Muller-Doblies et al., 2004), has practical implications. BRV, BCV and BRSV, observed to provoke subclinical infections in the second half period of the study did not lead to an activation of the IFN-α/β system. This mirrors the growing evidence for the ability of viruses to subvert the IFN-α/β system. This field study ultimately evidenced that recording of APRs will be a valuable tool to disclose critical circumstances that favour morbidity in different animal management systems.

6. Conclusion

APRs can be recorded by determining rising levels of APPs, such as Hp, SAA and Mx protein. Description of APRs during monocausal infections may define a profile of changes

characteristic of that pathology, which will assist in identifying the pathogenic mechanisms and aid specific diagnosis. Alternatively, measurement of the APR may reflect the severity of a disease process and serve as a prognostic indicator or, in the context of VPH, serve as a tool for the surveillance of animal health. The goal of the present field study was a successful inquiry into the feasibility of the later, i.e. animal health monitoring.

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